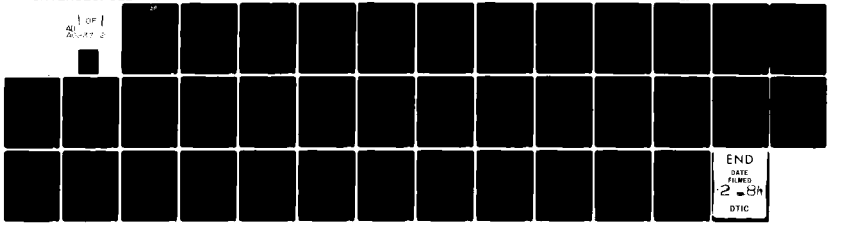
AD-A093 782 ARMY INST OF DENTAL RESEARCH WASHINGTON DC F/G 6/5
IDENTIFICATION OF LEUKOCYTE SUBPOPULATIONS RESPONSIBLE FOR THE --ETC(U)
UNCLASSIFIED DEC 80 C E HAWLEY 88-6039 NL



LEVEL II

AD A093782

BIBLIOGRAPHIC DATA SHEET	1. Report No. IAA YO1-DE-60025	2. AD-A093782	3. Recipient's Accession No.
4. Title and Subtitle: Identification of Leukocyte Subpopulations Responsible for the Production of "Osteoclast Activating Factor" and their Role in Bone Resorption.			5. Report Date: 31 December 1980
7. Author(s): Charles E. Hawley D.D.S., Ph.D.			8. Performing Organization Rept. No. OG-6039
9. Performing Organization Name and Address: U. S. Army Institute of Dental Research, Walter Reed Army Medical Center, Washington, DC 20012			10. Project/Task/Work Unit No. 00-040
			11. Contract/Grant No. YO1-DE-60025
12. Sponsoring Organization Name and Address: National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland 20205			13. Type of Report & Period Covered: Final Report
			14.

15. Supplementary Notes
None

16. Abstract: Osteoclast Activating Factor (OAF) was predictably produced in large volume mitogen stimulated MNC cultures. OAF production was independent of DNA synthesis.

OAF was produced in mixed leukocyte cultures containing heat inactivated fetal calf serum. OAF production was independent of blastogenesis.

OAF activity was isolated in the molecular weight range of 12,000 to 25,000 daltons by S-200 Sephacryl chromatography and high performance liquid chromatography (HPLC).

OAF activity was concentrated across membranes and by ammonium sulfate precipitation. Subsequent HPLC produced two peaks of bone resorbing activity: a major peak at 18,000 daltons and a minor peak at 9,000 daltons.

MNC culture supernatants also contained high concentrations of low molecular weight prostaglandins which also are believed to be osteolytic in vitro.

17. Key Words and Document Analysis. 17a. Descriptors
Bone Resorption
Lymphokines
Osteoclast Activating Factor

(9) Final rept. Oct 77-Sep 80

DTIC ELECTE JAN 14 1981 S D E

17b. Identifiers/Open-Ended Terms
Isolation and Purification

17c. COSATI Field/Group

038670

18. Availability Statement	19. Security Class (This Report)	21. No. of Pages
Release Unlimited	UNCLASSIFIED	
	20. Security Class (This Page) UNCLASSIFIED	22. Price

FORM NTIS-35 (REV. 3-72) THIS FORM MAY BE REPRODUCED USCOMM-DC 14952-P72

DTIC FILE COPY

Interagency Agreement No. YO1-DE-60025

IDENTIFICATION OF LEUKOCYTE SUBPOPULATIONS RESPONSIBLE FOR THE PRODUCTION OF "OSTEOCLAST ACTIVATING FACTOR" AND THEIR ROLE IN BONE RESORPTION

Charles E. Hawley, Colonel, Dental Corps
Division of Oral Biology
United States Army Institute of Dental Research
Washington, D. C. 20012

31 December 1980

Final Report for Period October 1977 to September 1980

Prepared For
National Institute of Dental Research
National Institutes of Health
Bethesda, Maryland 20205

TABLE OF CONTENTS

I. INTRODUCTION

A. History

The original proposal for research support entitled "Identification of Leukocyte Subpopulations Responsible for the Production of Osteoclast Activating Factor and Their Role in Bone Resorption" was submitted by Colonel John E. Horton, approved by the United States Army Institute of Dental Research (USAIDR) and the United States Army Medical Research and Development Command, and contracted through the Office of Collaborative Research, National Institute of Dental Research (NIDR) for services from 16 August 1976 to 30 September 1977. During that one year and six week period, Dr. Horton worked in collaboration with co-workers at the laboratory of Microbiology and Immunology, NIDR, to test procedures that would produce large quantities of OAF and to test procedures designed to isolate and purify bone resorbing factors in leukocyte culture supernatants. The results of this activity appear in the manuscript entitled "Partial Purification of a Bone Resorbing Factor Elaborated from Human Allogeneic Cultures" which appeared in Cellular Immunology 43:1-10, 1979 (Horton, et al). In April 1977 it was apparent that Dr. Horton would be unable to continue as principal investigator because of his retirement from active military duty. It was suggested by Colonel Duane E. Cutright, Commander, USAIDR, that Colonel Charles E. Hawley continue as principal investigator. Subsequently, a meeting was held on 22 April 1977 between representatives of NIDR and the USAIDR to discuss the future of the Interagency Agreement. It was decided that a revised proposal be submitted by Dr. Hawley for continuation of the contract after 1 September 1977. Because of basic differences in the research backgrounds of Dr. Horton and Dr. Hawley, it was requested that the new proposal include impact statements related to the time required to familiarize Dr. Hawley with the technical aspects of the project, any deviation in the direction of research outlined in the original agreement, and the additional time required to complete the proposed research. The final decision to continue the agreement between NIDR and the USAIDR would be made by the Project Officer, Dr. Stephen E. Mergenhagen. On 6 May 1977, a proposal for continuation and completion of the Interagency Agreement YO1-DE-60025 was presented to NIDR for approval. The new proposal outlined a six month transition period (1 July 1977 to 31 December 1977) during which the new principal investigator, working at a time commitment of 40%, would develop the independent expertise necessary for continuation of the project. This included learning the leukocyte culture techniques and the bioassay for bone resorption utilized by Dr. Horton. The proposal also requested that the Research Chemist, GS-12, be employed as soon as possible by the Civilian Personnel Office, Walter Reed Army Medical Center. In addition to the six month transition period, an additional fifteen months of research support was requested in order to complete the objectives of the revised proposal. The proposal was approved as noted in the letter from Dr. J. F. Goggins, Associate Director for Collaborative Research, NIDR, to Colonel Duane E. Cutright, Commander, USAIDR, dated 31 May 1977. The amended Interagency Agreement specified that contracted services begin on 16 August 1977 and end on 31 March 1979. Dr. Hawley's appointment as Principal Investigator was effective 1 September 1977. At that time, two military researchers, following the guideline of a 40% time commitment, became proficient with the culture techniques basic to the proposed research. Also during this time, negotiations continued for hiring the two full-time personnel listed on the original proposal. It was

not until 17 April 1978 that the biochemist position was filled. The biological laboratory technician was on duty on 1 July 1978.

Because of the delays in acquiring the chemist and laboratory technician, progress during 1978 was reflected largely in perfecting the bone resorption assay and the methods for the production of large volumes of OAF-containing supernatants. In addition, the initial steps of OAF purification, i.e., ultrafiltration and gel filtration, were developed. Details of progress from 1 October 1977 to 30 September 1978 appeared in the progress report from USAIDR to NIDR dated 20 December 1978. This report also contained a proposal for an extension of support from 1 April 1979 to 30 September 1980, and included the salary of an additional laboratory technician. On 2 February 1979, a site visit team from NIDR comprised of Dr. Stephen E. Mergenhagen, Dr. Clarence L. Trummel, Dr. Hyun Shin, and Dr. Robert J. Genco, reviewed the proposal and the USAIDR research facilities in Building 120A, Forest Glen Section, Walter Reed Army Medical Center. The proposal was extended to 30 April 1979 on 9 April 1979, and finally, on 18 May 1979, was extended to 30 September 1980. Dr. Stephen Mizel replaced Dr. Mergenhagen as NIDR project officer. At this time, USAIDR requested the Civilian Personnel Office, WRAMC to recruit and hire the additional full-time technician. This vacancy in the contract was not filled until 8 October 1979. On 24 August 1979, Colonel Charles E. Hawley, the principal investigator, was appointed Chief, Division of Oral Biology. This necessitated a physical relocation from Forest Glen to Washington, DC and precluded participation at the originally proposed 40% level.

A progress report for FY 1979 (including data obtained during October, November, and December 1979) was submitted to NIDR on 25 January 1980. On 25 May 1980, the research chemist appointment expired, and acting on the recommendations of Dr. Mizel and Dr. Mergenhagen, this appointment was not renewed. Two weeks later, on 13 June 1980, one of the full-time laboratory technicians left USAIDR for permanent employment with the Department of Agriculture. Since there were no plans to renew the agreement beyond 30 September 1980, this position was not refilled. Productivity from 1 April 1979 and 30 September 1980 was further aggravated by two periods of prolonged absences (illness in Greece) involving the research chemist.

B. Special Military Relevancy

This proposed research activity is consistent with the USAIDR mission of developing treatment methods which will decrease the morbidity of combat wounds. Combat wounds of the maxillofacial region frequently involve the loss of osseous tissue. These wounds are often contaminated with environmental bacteria which are major etiologic factors in chronic osteomyelitis and other granulomatous diseases. Current treatment employed in the correction of these processes may include resection procedures which contribute to delays in healing, prolonged convalescence, and, in some cases, permanent deformity. To counter these unwanted sequelae, therapeutic measures are required which will shorten treatment time and return the wounded soldier to duty without functional limitations. Currently, the replacement of lost osseous tissue using biodegradable ceramics has produced encouraging results in studies performed at USAIDR (Bhaskar, et al, 1971; Levin, et al, 1974). However, if these and other bone or tooth replacement materials are to be justified, it will be essential to first understand the mechanisms of bone pathology and,

in particular, the factors involved in osteolytic processes. Knowledge of basic concepts will enable investigators to design and evaluate bone replacement methods which will be backed by sound scientific criteria. Using an *in vitro* correlate of bone resorption, we have already produced evidence to suggest that the biodegradable ceramic, tricalcium phosphate, will inhibit the anticipated parathyroid hormone stimulated release of ^{45}Calcium from bony explants. While the mechanism of this inhibition is not known, we feel that the activity of other biologic mediators of bone resorption (OAF, prostaglandin E_2) may be similarly affected by tricalcium phosphate in our bioassay for bone resorption. In addition, the potential availability of the proposed anti-OAF antibody offered the intribuing opportunity for OAF neutralization studies *in vitro*, the potential for OAF neutralization *in vivo*, and the possibility of presenting evidence for OAF localization *in situ*. These studies might have further shown that the inhibition of biologically determined bone resorption might favor accelerated wound healing through enhanced bone deposition, and a biologic rationale for bone augmentation would have been supported.

C. Review of Pertinent Literature

There is ample evidence to suggest that the reactions of cell mediated immunity are important to the immunopathology of periodontal disease (Ivanyi, Wilton, and Lehner, 1972; Horton, Oppenheim, and Mergenhagen, 1974; Mackler, et al., 1974; Paters, et al., 1976; Lang and Smith, 1977; Seymour, Powell, and Davies, 1979). The mechanisms for tissue destruction are believed to be effected through the production and release of lymphokines from activated lymphocytes. These mediators may be largely responsible for the cellular events seen in the marginal lesion of periodontal disease (Page and Schroeder, 1976). One of these products of activated lymphocytes, osteoclast activating factor (OAF), has been detected in the supernatants of mitogen and antigen stimulated leukocyte cultures (Horton, et al., 1972; Trummel, Mundy, and Raisz, 1975) as well as mixed leukocyte cultures (Horton, et al., 1979). OAF may be responsible for the osteolytic processes observed in periodontal diseases and other granulomatous processes. OAF production in vitro is dependent upon the interaction of monocytes and lymphocytes in culture (Horton, et al., 1974), but OAF correlates in vivo and the lymphocyte subpopulation(s) involved in OAF production have not been identified.

Initial isolation and characterization studies showed that OAF activity fell within a molecular weight range of 13,700 to 25,000 daltons, and that its bone resorbing activity in vitro could be separated from that of other recognized osteolytic factors including parathyroid hormone, prostaglandin E_2, and active metabolites of Vitamin D (Luben, et al., 1974). Another report suggested that bone resorbing activity could be detected in leukocyte culture supernatants between chymotrypsinogen (25,000 daltons) and cytochrome C (12,500 daltons) and/or between calcitonin (3,500 daltons) and Vitamin B_{12} (1330 daltons) representing "big" OAF and "little" OAF, respectively, in a freely associating and disassociating, ionic strength dependent, subunit system (Mundy and Raisz, 1977). OAF isolation to date has progressed to the identification of an active bone resorbing peptide which appears as a single band on SDS-urea gel electrophoresis and has a molecular weight of 9,000 daltons (Luben, 1978). This 9,000 dalton species of bone resorbing activity may be the basic monomeric subunit of the OAF activity seen at 18,000 daltons following Sephadex G-100 chromatography (Luben, et al., 1974) and the OAF activity eluted from Sephadex G-75 in the molecular weight range of 11,000 to 18,000 daltons (Horton, et al., 1979).

Recently, Luben reported the hybridoma production of a monoclonal monospecific antibody that neutralized the effect of OAF in vitro (Luben et al., 1979) and later presented a rational approach for the isolation and purification of OAF in biologic fluids by ultrafiltration and polyacrylamide gel electrophoresis (Luben, 1980). These findings underscore the potential for the identification and localization of OAF in situ.

D. Purpose and Specific Aims

This document is the final comprehensive technical report of progress in the Interagency Agreement YO1-DE-60025 entitled "The Identification of Leukocyte Subpopulations Responsible for the Production of

Osteoclast Activating Factor and Their Role in Bone Resorption." The specific aims of the project were:

- To examine leukocyte culture variables including: the mitogen employed to stimulate OAF production, the culture media, the volume of the cultures, and the source of leukocytes placed in culture, in order to identify those conditions which will contribute to the most efficient production of OAF.

- To isolate and purify OAF according to various techniques, both published (Luben, et al., 1974; Raisz, et al., 1975; Mundy and Raisz, 1977; Luben, 1978; and Horton, et al., 1979) and unpublished, and to confirm the characterization studies reported previously (Raisz, et al., 1975).

- To produce antibody in experimental animals against the purified OAF preparation, and to confirm the specificity of the anti-OAF antibody by neutralizing the effect of OAF in the bioassay for bone resorption and by demonstrating the serologic reactivity of the antibody with OAF.

- To identify the leukocyte subpopulations responsible for the production of OAF.

II. PROGRESS REPORT

A. Bioassay For Bone Resorption

The major technical activity under the agreement during the period from 1 September 1977 to 31 December 1977 was directed toward the familiarization of the newly designated principal investigator and a biological science technician with the mechanics required for successful management of the bioassay for bone resorption (Raisz and Niemann, 1969). Briefly, this assay for bone resorption *in vitro* is an organ culture system which measures the release of ^{45}calcium from paired shafts of the radius and ulna dissected from 19-day rat fetuses. After a 24-hour equilibration period, the bony diaphyses are transferred to the control and experimental media and incubated for 120 hours at 37C in 5% CO_2. The culture media employed in these organ cultures is a modified BGJ medium supplemented with 1 mg/ml bovine serum albumin, fraction V; 1 mM L-glutamine; 100 units of penicillin; and 100 µg streptomycin. The amount of bone resorption is determined by liquid scintillation spectrophotometric analysis of the release of ^{45}calcium into the media at 48 and 120 hours versus the amount of radioactivity remaining in the explants at the end of the incubation period. Data are expressed as mean per cent release of ^{45}calcium from the bones as well as the mean ratio of treated to control bone cultures (T/C). These trials produced interexaminer precision in T/C ratios from unstimulated cultures of 1.01 ± .03 (n=24) and intraexaminer precision in PTH stimulated cultures of 1.98 ± .17 (n=11) and 1.75 ± .15 (n=11). The effect of PTH was significant ($p<0.01$) as anticipated from previously reported work with this bioassay (Luben *et al.*, 1974; Raisz *et al.*, 1975). Because of personnel changes that occurred in FY 1979 and FY 1980, it was necessary, from time to time, to train five new technicians in the bioassay. Data produced by these personnel was also highly predictable and reflected both interinvestigator and intrainvestigator precision.

B. OAF Generation in Human Peripheral Mononuclear Leukocytes

1. Concanavalin A (Con A):

Normal human peripheral leukocytes were obtained from the Walter Reed Army Medical Center Blood Bank. These cells are normally discarded in the preparation of leukocyte-poor packed erythrocytes. Mononuclear cells were prepared by sedimentation at 1xG for 90 minutes and separated on Ficoll-Hypaque isopyknic gradients (Boyum, 1968). Mononuclear cells (1 x 10^6/ml) were cultured in one dram vials with RPMI 1640 medium that was supplemented with 0.5% autologous plasma, 50 units penicillin/ml, and 50 µg streptomycin per ml. The cultures were incubated in 5% CO_2 in air at 37C for 96 hours. The 1 ml cultures were stimulated with 10 µg of the jack bean lectin, Con A, after the first 24 hours of incubation. Con A was selected for study because of the adsorption characteristics this material has to Sephadex, which could be of advantage in the OAF purification protocols (Trummel, Mundy, and Raisz, 1975). The cultured mononuclear cells were separated by centrifugation at 200 x G, and the harvested supernatants were tested for OAF activity in the bioassay for bone resorption.

2. Pokeweed Mitogen (PWM):

A study comparing the effectiveness of the plant lectins, PWM, and Con A as stimulants for the production of OAF was performed using the small volume mononuclear leukocyte culture system. Mononuclear cells obtained from a single bleeding, and prepared as previously described, were employed to test the two mitogens. The MSI for the Con A stimulated cell culture was 73.3 while it was 53.3 for the PWM stimulated cells.

The supernatants were tested for bone resorbing activity in the bioassay for bone resorption. While the MSI were similar for both mitogens, only Con A was shown to stimulate significant bone resorbing activity. These data suggest that Con A may be superior to PWM for the production of OAF in mononuclear cell cultures ($p<0.01$).

3. Phytohemagglutinin (PHA):

We also investigated the ^3H TdR uptake and the OAF generating capacities of PHA, and compared the results with those produced by Con A. Human mononuclear cells were obtained by either leukapheresis or plateletpheresis (Plateletpheresis Center, National Institutes of Health) and placed in the small 1 ml culture systems at 1×10^6 cells per ml. Paired cultures were stimulated after 24 hours with either PHA (1% final concentration) or Con A (10 μg/ml). The supernatants were collected 72 hours later and the cell pellets were pulse labeled with ^3H TdR. The ^3H TdR uptake results indicate that the mitosis stimulation index produced by PHA was generally greater than that produced by Con A (mean ratio PHA/Con A = 4.98) or that there was a 500% difference between the two mitogens in stimulating blastogenesis.

The OAF activity determinations suggest that PHA may be a more effective stimulant for the production of bone resorbing activity in the mononuclear cell cultures. However, when a statistical analysis is made of the mean T/C ratios produced by the large volume Con A (1.77 ± 0.17) and PHA (1.91 ± 0.30) stimulated supernatants, there is clearly no statistical difference in the OAF generating capacity of these two mitogens ($0.70>p>0.50$). While there are other trends displayed in the preceding data, i.e., the mean T/C ratios are higher for the large volume Con A stimulated cultures than for the respective parallel 1 ml small volume cultures (1.77 versus 1.39), these findings are only marginally significant ($0.10>p>0.05$). Similar suggestive differences are noted between the large and small volume PHA stimulated cultures. If only the Con A and PHA cultures that produced significant bone resorbing activity ($p<0.01$) are evaluated in the t test, the significance of difference between the activity in the large and small volume supernatants is improved to $0.05>p>0.01$. Figure 1 graphically shows the distribution of large and small volume supernatant T/C ratios obtained from 15 Con A and PHA stimulated mononuclear cell cultures. Since all but one of the points of the scattergram are located to the right (large volume side) of the ideal positive correlation line ($r = 1.00$), it is concluded that the large volume supernatants will predictably display higher bone resorbing activity over the small volume supernatants in the bioassay for bone resorption. We are now generating 500 ml to 2.5 liters of OAF-containing

supernatants from PHA stimulated, plasmafree, mononuclear cell (MNC) cultures using plateletpheresis preparations. This approach precludes the Ficoll-Hypaque step in MNC separation as the literature indicates that similarly prepared cell pellets contain 95% mononuclear cells (Mundy and Raisz, 1977). A report which evaluated Con A and PHA as stimulants for OAF production was presented at the 1979 meeting of the International Association for Dental Research in New Orleans, LA (Wyan, Hawley, and Kakari, 1979, Appendix I).

4. OAF Production in Mixed Leukocyte Cultures (MLC).

It was reported recently that high yield OAF-containing supernatants have been produced in human mixed leukocyte cultures (Horton, et al., 1979). In response to this paper and the queries raised during renewal of this inter-agency agreement in 1979, we investigated OAF production in MLC using mono-nuclear cells of plateletpheresis origin. The MLC's would have the potential advantage of yielding supernatants with bone resorbing activity without mitogen. These supernatants free of extraneous proteins might facilitate the isolation and purification of OAF.

PHA-stimulated MNC cultures and mitogen-free MLC were incubated in RPMI 1640 media alone or media modified with 0.5%, 1.0% or 2.0% fetal calf serum (FCS) or heat inactivated fetal calf serum (FCS*) (Farrar, J.J., 1974 and Farrar, J.J., 1975). Supernatants were tested for OAF activity by determining the ratio of test/control (T/C) per cent ^{45}Ca release and blastogenesis by ^{3}H TdR uptake in stimulated and unstimulated cultures (MSI). The mean and standard errors of MSI and T/C ratios for MNC cultures were 37.53 ± 3.58 and 1.86 ± 0.15, respectively. The mean MSI and T/C ratios for MLC without FCS were 2.84 ± 0.96 and 1.10 ± 0.05. With the addition of 0.5%, 1.0%, and 2.0% FCS, the mean MSI's were 3.30 ± 0.80, 4.60 ± 1.63, and 4.00 ± 0.63. Corresponding mean T/C ratios were 1.08 ± 0.06, 0.93 ± 0.04, and 1.20 ± 0.12. The mean MSI for MLC with 0.5%, 1.0%, or 2.0% FCS* were 5.44 ± 1.62, 4.20 ± 1.02 and 8.25± 2.53. The corresponding T/C ratios were 1.59 ± 0.10, 1.72 ± 0.12, and 1.75 ± 0.12. The t test showed no statistical difference ($p>0.10$) between the MSI of MLC using either FCS or FCS*. However, the OAF activity was higher ($p<.005$) when FCS* was used. These results show that OAF was produced only in plasma-free mitogen- stimulated MNC cultures or in MLC containing FCS*. These data also suggest that OAF production in MLC, as it was seen in mitogen stimulated MNC cultures, was independent of blastogenesis.

The resulting OAF activity in the crude supernatants obtained from MLC was not significantly different from that produced in the FCS-free mitogen-stimulated MNC cultures. Since FCS seems to be essential for OAF generation in MLC, and since the presence of serum proteins in culture supernatants might complicate OAF purification, the use of MLC for the production of bone resorbing activity has been abandoned.

An abstract of research describing the effect of FCS on the production of OAF in mixed leukocyte cultures was presented at the 1980 meeting of the International Association for Dental Research in Los Angeles, CA (Lamb, Hawley, and Kakari, 1980, Appendix II).

The acknowledged effectiveness of Con A in the stimulation of OAF production was detectable (T/C 1.56 ± .07, n=44) in the bioassay for bone resorption (Trummel, Mundy, and Raisz, 1975). During this phase of study, we observed that some mononuclear cell culture supernatants showed insignificant bone resorbing activity, while there was significant uptake of tritiated thymidine (^{3}H TdR) by the corresponding cell pellet. Because our isolation/purification procedures would require large volumes of high yield OAF-containing supernatants, and because previous studies suggested that the incorporation of ^{3}H TdR and the production of OAF may be unrelated events (Trummel, Mundy, and Raisz, 1975), we examined the relationship of OAF production in the small and large volume cultures as well as the uptake of ^{3}H TdR as a possible correlate to the OAF activity in culture supernatants. Having such a correlate might eliminate the need for testing the crude supernatants in the bioassay for bone resorption prior to the isolation of the OAF activity.

Mononuclear cells obtained by leukapheresis were maintained for 96 hours in either the small volume (1 ml) Con A (10 µg/ml stimulated cultures as previously described, or in parallel large volume (>200 ml) Con A stimulated cultures. Large volume supernatants were separated from the cultured leukocytes using a 0.45 µ filter. Only the small culture cell pellets were pulsed with ^{3}H TdR, and the incorporation of ^{3}H TdR by the stimulated and the unstimulated cells was measured by liquid scintillation spectrophotometry. The ratio of stimulated to unstimulated uptake of ^{3}H TdR, mitosis stimulation index (MSI), ranged from 1.18 to 273.0. When the culture supernatants were evaluated in the bioassay for bone resorption, mean test over control per cent ^{45}Ca release (T/C) ratios ranged from 0.88 to 1.85 (mean 1.33 ± .14, n=7) in the 1 ml cultures, and from 0.85 to 2.28 (mean 1.70 ± 0.21, n=7) in the large volume cultures. The correlation coefficient between ^{3}H TdR uptake and T/C in the small cultures was $r = 0.31$ ($p>0.10$) and $r = 0.64$ ($0.10>p>0.45$) in the large cultures.

There was a strong positive correlation, $r = 0.88$ ($p<0.01$), between the T/C ratios produced by the large and small volume supernatants. However, only the mean T/C ratio obtained by the large volume supernatants showed significant OAF activity ($p<0.01$). The t test further showed that there was no significant difference between the OAF activity found in the large and small volume supernatants ($0.20>p>0.10$). Since there was no significant correlation between the uptake of ^{3}H TdR and the production of OAF activity, and since some cultures showed measurable MSI without producing significant T/C ratios, it appears that the mitogenic response to Con A is quantitatively independent of the OAF activity in these cultures.

It would appear that the mitogenic response to Con A may be quantitatively independent of the OAF production in the respective cultures. Figure 2 shows the distribution of MSI versus T/C data (small volume supernatants only) data for 28 different mononuclear cell cultures. The cells were obtained from leukapheresis of plateletpheresis preparations and they were stimulated with either Con A, PWM, or PHA. An elevated MSI displayed by the cell pellet did not necessarily correspond to significant OAF activity in the culture supernatants. However, most of the bone resorbing activity was found in supernatants corresponding to cultures which produced MSI greater than 50.

C. Purification and Isolation

1. Concentration of Crude Supernatants.

Amicon stirred cells were used in conjunction with the UM-2 Diaflo membranes (1000 dalton molecular weight cut-off) for the concentration of MNC culture supernatants. The membranes were pretreated with a 1% aqueous solution of glycine to minimize the nonspecific binding of proteins to the membrane.

Serial dilutions of the crude MNC culture supernatants produced dose-response profiles of the OAF activity. Unfortunately, the bone resorbing activity apparent in the crude material sometimes became obscured during the concentration process. The activity obscured during concentration was repeatedly recovered in subsequent purification steps which suggests that the loss of bone resorbing activity was a function of concentrating the supernatants and not a function of membrane binding or rupture. The factors which might contribute to the disappearance of bone resorbing activity during concentration will be addressed in Section II.C.4.

We have investigated the use of PM-10 Amicon membranes which seemed more time efficient than the UM-2 membranes (Luben et al., 1974). The PM-10 membranes have a rated 10,000 dalton molecular weight cut-off and could have been helpful in the isolation of high molecular weight bone resorbing activity in mononuclear leukocyte culture supernatant concentrates (Luben, R.A. 1978). We have not been able to duplicate these filtrate findings in our laboratory. Table I shows the results of three experiments which tested for bone resorbing activity in crude supernatants, reconstituted PM-10 concentrates, and the corresponding PM-10 filtrates. Significant OAF activity was present in all PM-10 membrane filtrates tested. This suggests that the PM-10 membrane system is prone to compromise optimal OAF recovery in concentrates. Following discussions on the subject with the Project Officer, we terminated the use of PM-10 membranes for the purpose of concentrating mononuclear cell culture supernatants.

TABLE I. Bone Resorbing Activity in Crude MNC Supernatants, PM-10* Concentrates, and PM-10 Filtrates.

	Crude Supernatants	Reconstituted Concentrate	Filtrate
Batch 4	3.13 ± .83†	2.12 ± .35	1.84 ± .28
Batch 5	2.36 ± .32	0.97 ± .10	1.67 ± .33
Batch 7	1.45 ± .11	1.14 ± .20	1.71 ± .40

*PM-10 Membrane, Amicon Corporation
†T/C ratios of per cent ^{45}Ca released from bone explants.
(n=4 explant pairs)

2. Gel Filtration.

Sephacryl S-200 (Pharmacia) was used for gel filtration of UM-2 membrane concentrated MNC culture supernatants. Columns (90 cm X 2.5 cm) were packed at 4C and equilibrated with tris-Hcl buffer, pH 7.5 (0.05 M tris-HCl, 0.1 M NaCl) containing 50 μg/ml gentamycin. Columns were calibrated with blue dextran (molecular weight 2 X 10^6 daltons), aldolase (158,000 daltons), albumin (67,000 daltons), ovalbumin (43,000 daltons), myoglobin (18,800 daltons, and RN'ase (13,700 daltons). The effluent was monitored at 280 nm. Fractions were also evaluated at 220 nm because of the reported greater sensitivity of peptide bond structure at this wavelength. After placing a mixed pool of MNC culture supernatant concentrate (containing 0.5% autologous human sera) on the S-200 Sephacryl column, fractions were dialyzed in RPMI 1640, diluted 1:5 in bone resorption assay. Peak 280 nm absorbance was observed between aldolase (158,000 daltons) and ovalbumin (43,000 daltons) in fractions 15 to 25. Bone resorbing activity (T/C = 2.06 ± .34) was observed in the dialyzed fractions suspected of containing serum albumin (fraction 21). The basis of this serum protein associated bone resorbing activity has not yet been resolved. It may, however, have been due to non-specific binding of OAF to plasma proteins (albumin) as Luben and coworkers have observed the elution of bone resorbing activity with albumin on S-150 Sephadex columns (Luben, et al., 1974). In addition, other peaks of OAF-like activity were present in RPMI dialyzed fractions in the molecular weight range of slightly greater than myoglobin at 18,800 daltons (fraction 29) to slightly below RN'ase at 13,700 daltons (fraction 34). The OAF-active fractions containing proteins with molecular weights less than 43,000 daltons did not show significant ultraviolet absorbance at 280 nm but protein absorbance was observed at the 220 nm wavelength. Neither of the resulting absorbance profiles paralleled the distribution of OAF activity of the column effluent. Low molecular weight bone resorbing activity was also found in fractions 35 through 38 and in fractions 43 through 54 estimated to contain proteins in the molecular weight range of less than 10,000 daltons. These fractions may contain the low molecular weight 9,000 dalton species of OAF (Luben, 1978), and the "little OAF" moiety between 3500 and 1330 daltons (Mundy and Raisz, 1977). Figure 3 graphically depicts an S-200 Sephacryl elution profile of a single run of pooled UM-2 concentrated OAF-containing, cell-free, leukocyte culture supernatants, and a mean per cent Ca release T/C ratios which describe the bone resorbing activity found in the effluent fractions. Subsequent retests of these S-200 Sephacryl fractions for bone resorbing activity have been performed. The results are shown in Table II. Bone resorbing activity was again noted in fractions believed to contain serum albumin (fraction 21), and in the fractions suspected of containing proteins in the molecular weight range of 13,000 to 20,000 daltons. Chromatography of other crude supernatant concentrated on S-200 Sephacryl has produced similar distribution of OAF activity.

Subsequently, we investigated the use of High Performance Liquid Chromatography (HPLC) for the purification of OAF activity in S-200 fractions in the the estimated molecular weight range of 5,000 to 35,000 daltons. An analytical HPLC column, kindly provided *gratis* by Waters Associates Inc., was

TABLE II. Bone Resorbing Activity Eluted by S-200 Sephacryl Chromatography

Fraction*	Test/Control Ratio Per Cent Release ^{45}Ca	Number of Explant Pairs Tested
18	1.26 ± 0.15†	n = 8
21	2.04 ± 0.42	n = 8
27	1.04 ± 0.09	n = 8
29	1.54 ± 0.20	n = 16
31	1.36 ± 0.13	n = 8
33	1.08 ± 0.06	n = 8

*Dialyzed in RPMI 1640, diluted 1:5 in BGJ/1% FCS
†Mean ± Standard Error

standardized with aldolase at 158,000 daltons, ovalbumin at 45,000 daltons, myoglobin at 18,800 daltons, and RN'ase at 13,700 daltons (Figure 4) and equilibrated with tris-HCL buffer, pH 7.5. A concentrated (150X at 3 mg total protein/ml fraction pool of OAF active S-200 fractions was applied (30 µl), and 1 ml fractions were collected. HPLC fractions were combined in three pools, and the undialyzed pools were tested in the bone resorption assay. The results of this testing are shown in Table III.

TABLE III. Bone Resorbing Activity of Pooled S-200 Sephacryl Fractions*

	T/C Ratio Per Cent Release ^{45}Ca	
Fraction Pool	1:2 Dilution in Media†	1:5 Dilution in Media†
A (14, 15, 16)	0.95 ± .01‡ (n = 4)	0.85 ± .13
B (17, 18, 19)	1.89 ± .14 (n = 4)	2.43 ± .23
C (20, 21, 22)	1.57 ± .18 (n = 4)	1.05 ± .03

*Estimated molecular weight range of 8,000 to 25,000 daltons.
†BGJ supplemented with 1% FCS
‡Mean ± Standard Error

The pool of fractions 17, 18, and 19 (B), estimated to contain protein with a molecular weight range of 20,000 to 18,000 daltons showed bone resorbing activity (T/C = 1.89 ± .14 and 2.4 ± .23) at 1:2 and 1:5 dilutions, respectively. The pool of fractions 20, 21, and 22, estimated to contain proteins with a molecular weight range of 18,000 to 14,000 daltons also stimulated the release of ^{45}Ca, but at the 1:2 dilution only. Testing of individual HPLC fractions (diluted 1:2) showed significant bone resorbing activity (T/C ratios) as follows: fraction 16, 0.90 ± .14; fraction 17, 2.44 ± .12; fraction 18, 1.54; fraction 19, 2:54 ± .39; fraction 20, 2.76 ± .31; fraction 21, 2.88; and fraction 22, 1:46 ± .18. Due to the small quantity of analytical material provided in these experiments, total protein and dose response profiles were not measured. These results do show, however, that when concentrated pooled active S200 fractions are analyzed by HPLC molecular sized exclusion chromatography, bone resorbing activity can be recovered in the molecular weight range of approximately 10,000 to 20,000 daltons. Figure 5 summarizes the results of testing HPLC fractions in the bone resorption assay.

3. Ammonium Sulfate Precipitation

Acting on the advice and counsel of the NIDR Project Officer, Dr. S. B. Mizel, and faced with the need of obtaining large volumes of high specific activity OAF preparations, we began investigating the use of high salt concentrations for the precipitation of proteins and the potential application of this approach for OAF purification.

The well established methods of ammonium sulfate precipitation were employed (Dixon, M., 1953). Crude MNC culture supernatants were pooled (10.0 µg protein/ml) and concentrated across the UM-2 membrane (54X) prior to 25%, 45%, 65%, 85%, and 100% ammonium sulfate precipitation. The results of testing the precipitated protein fractions in the bone resorption assay produced the following T/C per cent ^{45}Ca release values: a 2X RPMI 1640 reconstitute of the crude concentrate, 1.31 ± .10; 25% saturation with ammonium sulfate (1 µg protein/ml), 0.91 ± .31; 45% saturation (12.5 µg/ml), 0.99 ± .12; 65% saturation (22.5 µg/ml), 1.23 ± .12; 85% saturation (17.5 µg/ml), 1.55 ± .17; and 100% saturation with ammonium sulfate (9 µg/ml), 1.38 ± .26.

Table IV shows the distribution of the total protein in a crude MNC culture supernatant during the initial phases of supernatant concentration and OAF purification. There was a 70.93% reduction of the original supernatant protein during concentration and a 98.78% reduction of the concentrated protein by ammonium sulfate fractionation. The 85% and the 100% ammonium sulfate pellets, which represented 0.13% of the original protein, were the only ammonium sulfate fractions showing significant bone resorbing activity. The greatest OAF activity appeared in the 85% ammonium sulfate preparation (T/C = 1.55 ± .17). Since only 0.125 ml of the precipitate (17.5 µg/ml) was in the 0.250 ml bone resorption assay, this OAF activity was demonstrated at a concentration of 0.55 µg/ml of culture media. A dose response profile of this material was not determined.

TABLE IV. Analysis of Protein Concentration in a Crude MNC Supernatant, in its UM-2 Concentrate, and in the Ammonium Sulfate Fractions of its UM-2 Concentrate.

Sample	Concentration*	Volume (ml)	Total Protein (mgs)	Per Cent Protein Reduction
Crude Supernatant	.010	2000.00	34.4	--
UM-2 Concentrate	.250	40.0	10.0	70.93
25% Precipitate	.001	2.5	.0025	
45% Precipitate	.0125	2.15	.027	
65% Precipitate	.0225	2.15	.048	98.78†
85% Precipitate	.0175	1.60	.028	99.56
100% Precipitate	.009	1.80	.016	

*Bio-Rad Coomassie Blue Method (Bradford, 1976)

†Per cent of 10.0 mgs protein.

As we had done with the OAF active fractions obtained by S-200 Sephacryl chromatography, the 85% and the 100% ammonium sulfate precipitates (100 µl at 17.5 and 9.0 µg/ml, respectively) were chromatographed on a standardized and tris-HCl buffer equilibrated analytical HPLC protein column. The standards utilized were aldolase (molecular weight 158,000 daltons, fraction 13), ovalbumin (molecular weight 45,000 daltons, fraction 15), myoglobin (molecular weight 18,800 daltons, fraction 18), and RN'ase (molecular weight 13,700 daltons, fraction 21). Three fraction pools in the molecular weight range anticipated to include OAF (20,000 daltons, to less than 10,000 daltons) were tested in the bone resorption assay. The results appear in Table V. No OAF activity could be detected in the HPLC effluent of the 100% ammonium sulfate precipitate, but, from the 85% precipitate, significant release of ^{45}Ca from long bone explants was measured in two of the HPLC fraction pools.

Representative 1 ml HPLC fractions from these pools were then tested in the bone resorption assay and the initial results showed that peak bone resorbing activity could be detected in the effluent containing proteins with molecular weights near or below myoglobin (18,800 daltons).

Table VI shows the results of testing fractions obtained from a second standardized and tris-HCl equilibrated analytical HPLC column following the

TABLE V. Distribution of Bone Resorbing (OAF) Activity in Ammonium Sulfate Precipitates of a Crude MNC Supernatant Concentrate Following High Performance Liquid Chromatography*

Fraction Numbers (Pool)	Test/Control Per Cent Release ^{45}Ca 85% Precipitate	100% Precipitate
20 - 22 (I)	1.58 ± .13†(n = 4)‡	0.96 ± .13 (n = 4)
23 - 25 (II)	1.49 ± .04 (n = 4)	1.02 ± .08 (n = 4)
26 - 28 (III)	1.22 ± .11 (n = 4)	1.11 ± .17 (n = 4)

*Waters Associates Inc.
†Mean ± Standard Error
‡n = number of explant pairs

TABLE VI. Distribution of Bone Resorbing Activity (OAF) in Ammonium Sulfate Precipitates of a Crude MNC Supernatant Concentrate Following High Performance Liquid Chromatography*

Fraction Numbers	Test/Control Per Cent Release ^{45}Ca 85% Precipitate	100% Precipitate
18	ND	1.06 ± .12 (n = 4)
19	1.19 ± .06†(n = 4)‡	0.90 ± .05 (n = 4)
20	1.16 ± .08 (n = 4)	0.97 ± .12 (n = 4)
21	4.66 ± .43 (n = 4)	1.11 ± .09 (n = 4)
23	1.77 ± .23 (n = 4)	ND
24	2.04 ± .10 (n = 4)	1.09 ± .13 (n = 4)
25	2.10 ± .18 (n = 4)	ND
26	1.45 ± .17 (n = 4)	ND
27	1.04 ± .05 (n = 4)	ND

*Waters Associates Inc.
†Mean ± Standard Error
‡n = number of explant pairs

addition of 500 µl of the same 85% and 100% ammonium sulfate saturation precipitates. The results indicate that significant low molecular weight bone resorbing activity (approximately 9,000 daltons) was isolated by combined ammonium sulfate and HPLC fractionation methods, and in addition, there was the peak of high molecular weight OAF activity (18,000 daltons) as was previously shown by combined S-200 Sephacryl and HPLC (Figure 5). Figure 6 graphically shows a comparison of proteins absorbance and bone resorbing activity profiles produced by combined ammonium sulfate and HPLC methods. The absorbance profiles in Figures 5 and 6 reflect differences in sample preparation prior to HPLC. It is tempting to speculate that during the ammonium sulfate precipitation there was a dissociation of the high molecular weight OAF activity shown by combined HPLC and S-200 chromatography, and that the low molecular weight OAF activity displayed by combined ammonium sulfate and HPLC was the same as the 9000 dalton species isolated on sodium dodecyl sulfate-urea gels (Luben 1978). Because of the small volume of the HPLC fractions (1 ml), inadequate amounts of material were available for total protein analysis and specific activity determinations.

4. Partial Purification of Ultralow Molecular Weight Osteolytic Factors

Throughout these investigations, we observed that the reconstitution of UM-2 filtrates with culture media (BGJ_b or RPMI 1640) showed an inhibition (loss) of bone resorbing activity. To help resolve the issue of possible inhibitors in these preparations, we tested the UM-2 concentrates, the UM-2 concentrates reconstituted with the corresponding UM-2 filtrates and media, and the UM-2 filtrates alone, for OAF-like activity. The UM-2 concentrates by themselves again showed the inhibition of bone resorbing activity and the filtrates showed osteolytic activity which we had not previously detected, and which was sometimes greater than that seen in the crude supernatants from which they were derived. The UM-2 concentrates reconstituted with filtrates were similar in activity to their crude supernatants (indicating negligible binding of osteolytic material to the membrane during concentration) and the concentrates reconstituted with media showed the inhibition of calcium release shown by the concentrates alone. The data are shown in Table VII.

The presence of potent osteolytic activity in the UM-2 membrane filtrates is certainly consistent with the reported osteoclast stimulating properties of low molecular weight prostaglandins (Klein and Raisz, 1970; Luben, et al., 1974; Holtrop, Raisz, and King, 1978). In addition, prostaglandins (PGE_1 and PGE_2) have been shown to be essential elements for the synthesis of OAF by MNC (Yoneda and Mundy, 1979). Consequently, we tested representative, biologically active, UM-2 preparations for prostaglandins by radioimmunoassay (Orczyk and Behrman, 1972). The results showed high concentrations of PGE (1.8 - 2.2 ng/ml) in the UM-2 filtrates which represented virtually all of the PGE found in the corresponding crude culture supernatants. The UM-2 membrane cut-off (1000 daltons), the high concentration of PGE in our

TABLE VII. The Effects of Pooled MNC Culture Supernatants, UM2 Concentrates, UM-2 Filtrates, and their Reconstituted Products on Osteolytic Processes *In Vitro*.

	Test/Control Percent Release ^{45}Ca ± Standard Error				
Patch Number (Concentration Factor)	PHA Stimulated MNC Culture Supernatant Pools	UM-2 Ultra Filtration Concentrate	UM-2 Ultra Filtration Filtrate	UM-2 Concentrate Reconstituted With Filtrate	UM-2 Concentrate Reconstituted With RPMI-1640
B_{14} (95X)	1.30 ± 0.08 1.38 ± 0.08	0.51 ± 0.06	2.10 ± 0.28	1.42 ± 0.10	0.86 ± 0.11
B_{15} (60X)	1.38*± 0.09	0.37 ± 0.04	1.02 ± 0.14	1.43 ± 0.15	0.76 ± 0.04
B_{16} (100X)	1.30 ± 0.29	0.50 ± 0.09	1.37 ± 0.24	1.04 ± 0.15	0.27 ± 0.20
B_{17} (100X)	1.23 ± 0.27 1.04 ± 0.11 1.36 ± 0.32	0.68 ± 0.12	3.84 ± 0.48 2.34 ± 0.19	1.03 ± 0.12 1.71 ± 0.21	0.67 ± 0.10
B_{18} (100X)	1.09 ± 0.05	0.60 ± 0.05	1.78 ± 0.23	1.19 ± 0.16	0.58 ± 0.06
B_{20} (40X)	1.16*± 0.11	0.90 ± 0.13	1.84 ± 0.17	1.11 ± 0.23	0.64 ± 0.05

*Prefiltered across a PM-10 membrane prior to UM-2 concentration.

UM-2 filtrates, and corresponding bone resorbing data suggest that prostaglandins (MW 350-354) were responsible for some of the osteolytic activity in these cultures.

The depressed bone resorbing activity in the UM-2 concentrates remained a mystery, particularly since we could predictably isolate OAF chromatographically from this material. One possible explanation could be the presence of a high molecular weight (>1000 daltons) inhibitor of osteolysis. To investigate this possibility, we performed the following modification of a recently published technique for the isolation of OAF in biologic fluids (Luben, 1980). Active crude supernatants were pooled (1,180 ml) and concentrated to 50 ml across a PM-10 membrane (10,000 daltons cut-off). The retentate was mixed with an equal volume of 2M NaCl for one hour. The concentration process was then resumed to a retentate volume of 20 ml. The concentrate was filter dialyzed with five volumes of Dulbecco's phosphate-buffered saline (PBS, pH 7.2, MA Bioproducts) and further concentrated to 11 ml (107 X concentrate of original crude pool). The PM-10 filtrate and dialysate were combined and concentrated (107 X) in a similar fashion across a UM-2 membrane. The rationale for this approach was that the PM-10 membrane would remove high molecular weight substances (>10,000 daltons) and parathyroid hormone as suggested by Luben (1980). The 1M NaCl would dissociate any high molecular weight OAF (18,000 daltons) into one of the lower molecular weight species (Mundy and Raisz, 1977; Luben, 1978) and permit its passage through the membrane during filter dialysis. The UM-2 membrane would then retain the low molecular weight OAF and the final UM-2 membrane dialysis would remove lower molecular weight compounds (<1000 daltons) such as prostaglandins or vitamin D that might affect the assay.

The results showed that the PM-10 concentrate had no activity in the bioassay for bone resorption (T/C 1.08 ± 0.03) while the PM-10 filtrate was biologically active (T/C 1.48 ± 0.06). The subsequent UM-2 concentrate showed inhibition of bone resorption (0.78 ± 0.01) and the UM-2 filtrate displayed significant osteolytic activity (T/C 1.26 ± 0.08). Reconstitution experiments with the UM-2 concentrates and filtrates produced data which paralleled that previously discussed and shown in Table VII.

The UM-2 concentrate was chromatographed to isolate the suspected osteolytic inhibitor. A Sephacryl S-200 column (96cm X 2.5cm) was equilibrated with PBS, pH 7.2, and partially standardized with myoglobin (18,000 daltons), fraction 67, and cytochrome C (molecular weight 12,384), fraction 69. Fractions (5 ml) were pooled and tested for bone resorbing activity. The results were as follows: Pool I (fractions 61-64) T/C 1.04 ± 0.05; Pool II (fractions 65-68) T/C 0.90 ± 0.04; Pool III (fractions 69-72) T/C 0.89 ± 0.06; Pool IV (fractions 73-76) T/C 0.50 ± 0.02; Pool V (fractions 77-80) T/C 0.43 ± 0.03; Pool VI (fractions 81-84) T/C 0.95 ± 0.02; Pool VIII (fractions 85-88) T/C 2.24 ± 0.07; Pool VIII (fractions 89-92) T/C 2.34 ± 0.05; and Pool IX (fraction 93-96) T/C 2.53 ± 0.13. These data are shown graphically in Figure 7 and show the possible effect of an osteolysis inhibitor in Pools IV and V and the presence of potent bone resorbing activity in Pools VII, VIII, and IX. The poor resolving power of Sephacryl S-200 in the lower molecular

weight ranges has, to date, precluded further isolation of these inhibitors and resorbers. Nonetheless, until these factors can be separated further by other techniques, it is tempting to suggest that the combination of membrane ultrafiltration, dialysis in 1M NaCl, and gel filtration chromatography, used here have isolated OAF in its basic monomeric unit, and that this bone resorber may be similar to the 3,500 dalton species of OAF described by Mundy and Raisz (1977). The detection of an inhibitor of osteolysis in these preparations of MNC culture supernatants warrants further investigation and may help explain our inability to predictably demonstrate bone resorbing activity in our UM-2 concentrates.

5. Anti-OAF Antibody Production

No progress to report in FY 1980.

6. Collaborative Studies

In June 1979, the principal investigator contacted Dr. R. A. Luben, University of California, Riverside, California, regarding collaborative studies which would investigate the activity of his purified human tonsillar OAF preparation (Luben, 1978) and its neutralization by monoclonal antibodies against OAF (Luben, et al., 1979) in our bone resorption assay. Dr. Luben agreed with this proposal. However, the nonavailability of precious explants in our bone resorption assay, and the fact that we have not been able to exchange biologically active materials with Dr. Luben, have delayed any significant progress in this area. Our attempts to visit Dr. Luben in his laboratory in March 1980 (IADR Meeting) were unsuccessful. We have provided access to our bone resorption assay by researchers from the Microbiology and Immunology Laboratory at NIDR. We have tested dilutions of a partially purified colony stimulating factor (CSF) for bone resorbing activity in vitro. The resulting data indicated that, as supplied, this material did not stimulate the release of ^{45}Ca from our bony explants. Likewise, at the beginning of FY 1980, we also tested IgE stimulated mast cell supernatants for bone resorbing acitivity, and again, the results did not suggest the presence of measurable osteolytic factors.

7. Miscellaneous

At Dr. Mizel's suggestion, an attempt was made to determine the feasibility of using carboxymethyl (CM) cellulose cation exchange chromatography for purification of OAF. A 30 X 1.5 cm column was packed with CM-52 (Whatman), equilibrated with 0.1 M sodium acetate buffer, pH 5.0, according to Dr. Mizel's suggested protocol, and calibrated with bovine serum albumin (BSA). At pH 5.0, chymotrypsinogen A and cytochrome C were retained by the CM-cellulose, while BSA appeared in break-through fractions. Both of the bound proteins were eluted separately in 0 - 2.0 M NaCl gradient. Ten ml of the PHA in a 1.0M NaCl stock solution (the amount that would be added to 1,000 ml of culture and retained by a UM-2 membrane) was first dialyzed on a spectropore 3 membrane with 0.1M sodium acetate buffer at pH 7.0. After dialysis of the NaCl, the pH of the retentate was shifted to pH 5.0 with a few drops of 1 N acetic acid. The resulting heavy white precipitate was removed by centrifugation and the supernatant was applied to the CM-cellulose column. The column was washed with three column volumes of buffer (fractions collected) prior to starting linear 0-2M NaCl gradient. A very small absorbance peak (280 nm) appeared in the

break-through fractions and a very low broad peak of absorbance showed in the gradient fractions. The absorbance profile suggested that almost all the PHA protein was precipitated during the acidification step. A second experiment was performed to determine if the precipitation of PHA might also co-precipitate other proteins present in test solutions. Ten mg each of BSA, chymotrypsinogen A and cytochrome C were added to 10 ml of the PHA stock solution. This was dialyzed and acidified as described above. The precipitate was again removed by centrifugation and the supernatant applied to the CM-cellulose column. A sharp peak corresponding to BSA came through in the break-through and two peaks corresponding to chymotrypsinogen A and cytochrome C came through in the gradient at 0.15M and 0.46M NaCl, respectively.

These results suggest that the indicator proteins were not co-precipitated with PHA, and also suggest that OAF activity may also survive PHA precipitation. We subsequently assayed the CM-cellulose buffer in the bone resorption bioassay and discovered that the acetate buffer was incompatible with the system. In addition, since OAF activity has been reported labile below pH 6 (Luben, et al., 1974), the acidity of the acetate buffer required for CM-cellulose chromatography could inactivate any bone resorber present in test materials. The lability of OAF in acidic solutions, and the incompatibility of acetate buffers with the bioassay were significant in our abandoning further evaluation of CM-cellulose chromatography for OAF isolation.

During FY 1980 two technical problems developed which curtailed progress in OAF isolation experiments. During March and April 1980, the usual levels of bone resorbing activity disappeared from our large volumes of MNC culture supernatants. Trypan blue exclusion and ^{3}H TdR testing suggested that MNC viability was consistent with that previously shown capable of OAF production, and personnel at the NIH Plateletpheresis Center indicated that the plateletpheresis protocol had not been modified during the period in question. This problem was finally traced to a particular lot of RPMI-1640 from MA Bioproducts (Lot 97032). We later learned that at least one laboratory at NIDR had experienced similar difficulties culturing MNC with the same lot of media. From early June to mid-August 1980, our eight-year-old liquid scintillation counter was down with repeated malfunctions. The problems were probably caused by a local power surge at Forest Glen which caused extensive damage to electronic circuits and greatly complicated attempts at repair by the service representatives (Packard). The deteriorating reliability of this instrument ultimately resulted in a unit replacement in October 1980. Since this machine was necessary for determining the results of our bioassay for bone resorption, there was a two month backlog of samples for evaluation by liquid scintillation spectrophotometry. This forced suspension of some experiments, the termination of others, and a significant delay in submitting this final report.

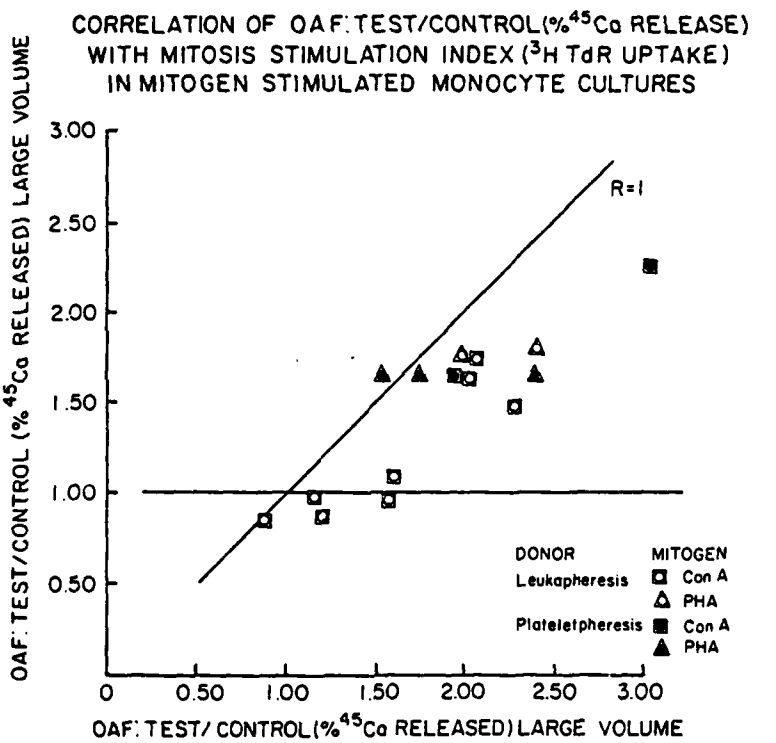

CORRELATION OF OAF: TEST/CONTROL(%45Ca RELEASE)
WITH MITOSIS STIMULATION INDEX (3H TdR UPTAKE)
IN MITOGEN STIMULATED MONOCYTE CULTURES
OAF: TEST/CONTROL (%45Ca RELEASED) LARGE VOLUME
3.00
2.50
2.00
1.50
1.00
0.50
0
0.50
1.00
1.50
2.00
2.50
3.00
R=1
DONOR
MITOGEN
Leukapheresis
Con A
PHA
Plateletpheresis
Con A
PHA
OAF: TEST/ CONTROL(%45Ca RELEASED) LARGE VOLUME

Figure 1.

CORRELATION OF OAF: TEST/CONTROL (%^{45}Ca RELEASE)
WITH MITOSIS STIMULATION INDEX (^{3}H TdR UPTAKE)
IN MITOGEN STIMULATED MONOCYTE CULTURES

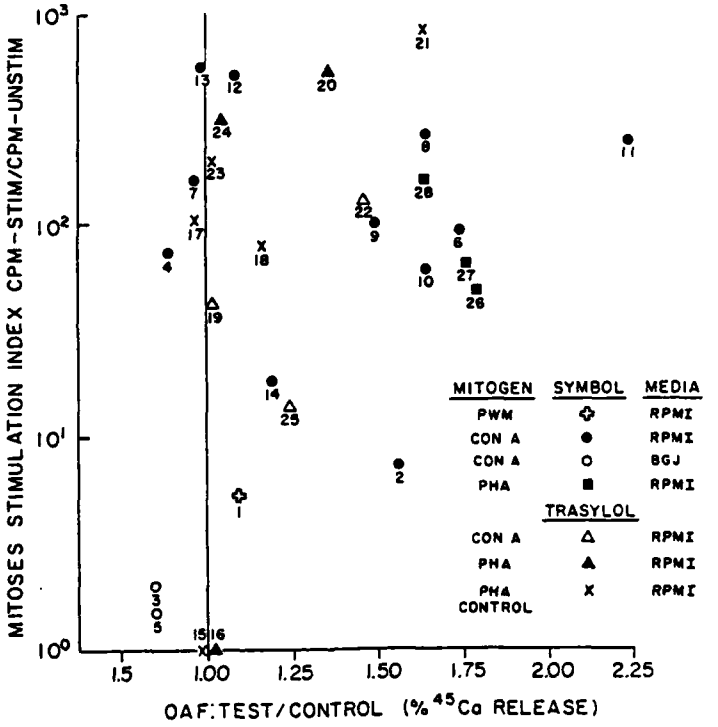


Figure 2.

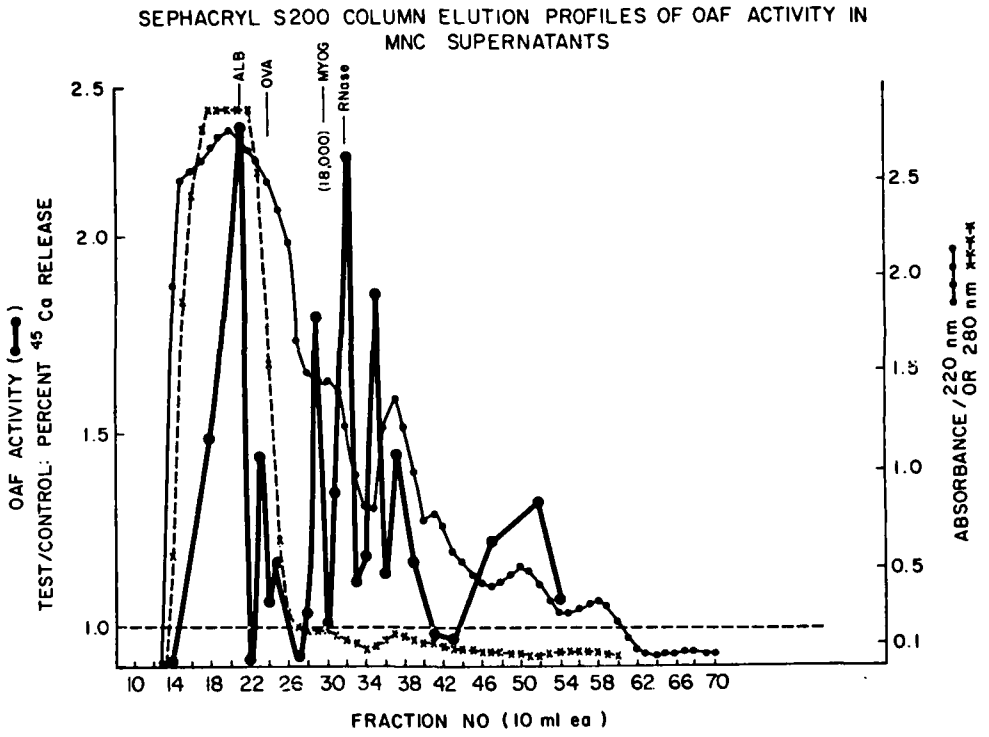

SEPHACRYL S200 COLUMN ELUTION PROFILES OF OAF ACTIVITY IN MNC SUPERNATANTS
ALB
OVA
(18,000) MYOG
RNase
OAF ACTIVITY (●—●) TEST/CONTROL: PERCENT 45Ca RELEASE
ABSORBANCE / 220 nm ●—●—● OR 280 nm ✱-✱-✱
2.5
2.0
1.5
1.0
0.5
0.1
10 14 18 22 26 30 34 38 42 46 50 54 58 62 66 70
FRACTION NO (10 ml ea)

Figure 3.

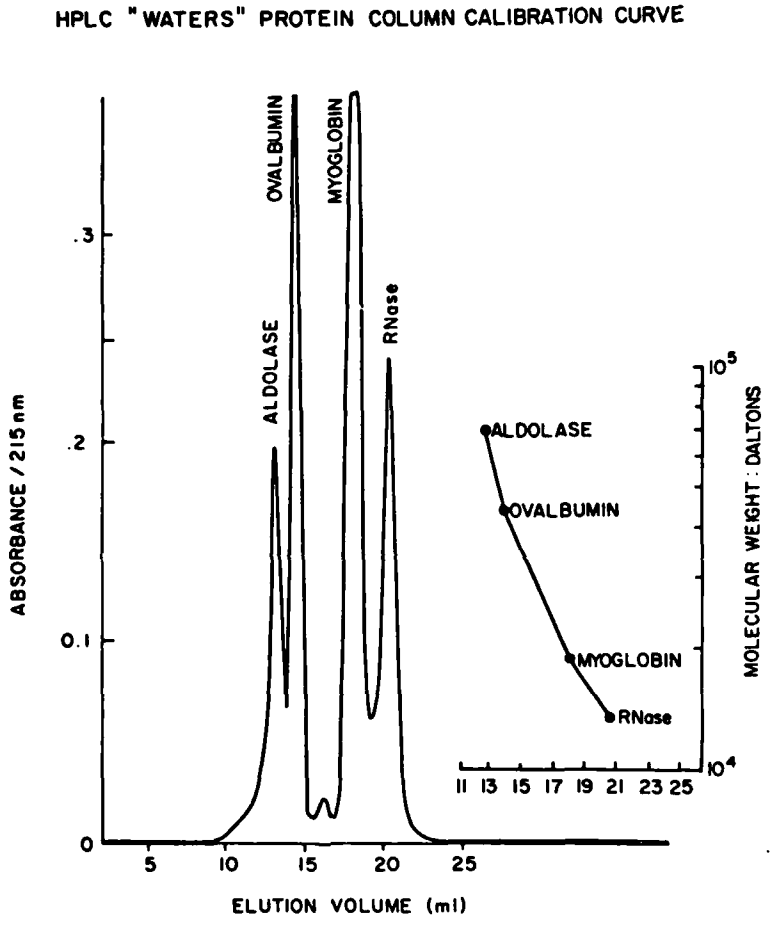

HPLC "WATERS" PROTEIN COLUMN CALIBRATION CURVE
ABSORBANCE / 215 nm
.3
.2
0.1
0
ALDOLASE
OVALBUMIN
MYOGLOBIN
RNase
5
10
15
20
25
ELUTION VOLUME (ml)
ALDOLASE
OVALBUMIN
MYOGLOBIN
RNase
10^5
10^4
11 13 15 17 19 21 23 25
MOLECULAR WEIGHT : DALTONS

Figure 4.

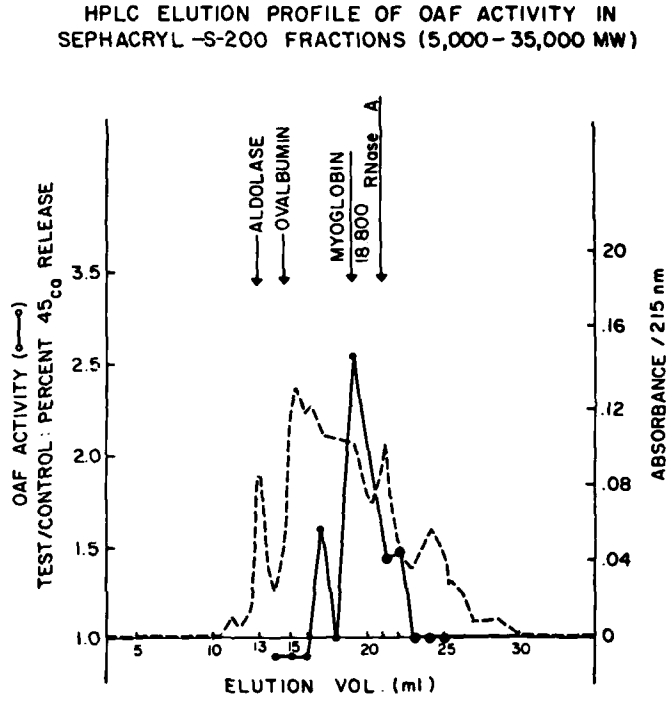

HPLC ELUTION PROFILE OF OAF ACTIVITY IN
SEPHACRYL -S-200 FRACTIONS (5,000 - 35,000 MW)
OAF ACTIVITY (o—o)
TEST/CONTROL: PERCENT 45Ca RELEASE
ABSORBANCE / 215 nm
ALDOLASE
OVALBUMIN
MYOGLOBIN
18 800
RNase A
3.5
2.5
2.0
1.5
1.0
20
.16
.12
.08
.04
0
5
10
13
15
20
25
30
ELUTION VOL. (ml)

Figure 5.

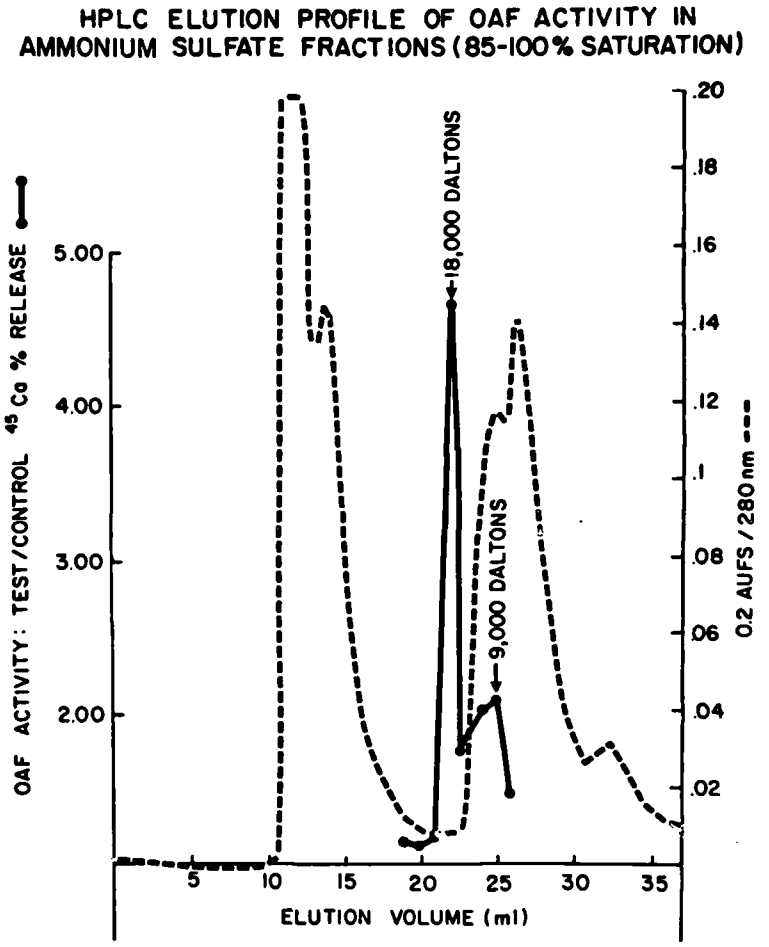

HPLC ELUTION PROFILE OF OAF ACTIVITY IN
AMMONIUM SULFATE FRACTIONS (85-100% SATURATION)
OAF ACTIVITY: TEST/CONTROL 45Ca % RELEASE
5.00
4.00
3.00
2.00
18,000 DALTONS
9,000 DALTONS
.20
.18
.16
.14
.12
.1
.08
.06
.04
.02
0.2 AUFS / 280nm
5
10
15
20
25
30
35
ELUTION VOLUME (ml)

Figure 6.

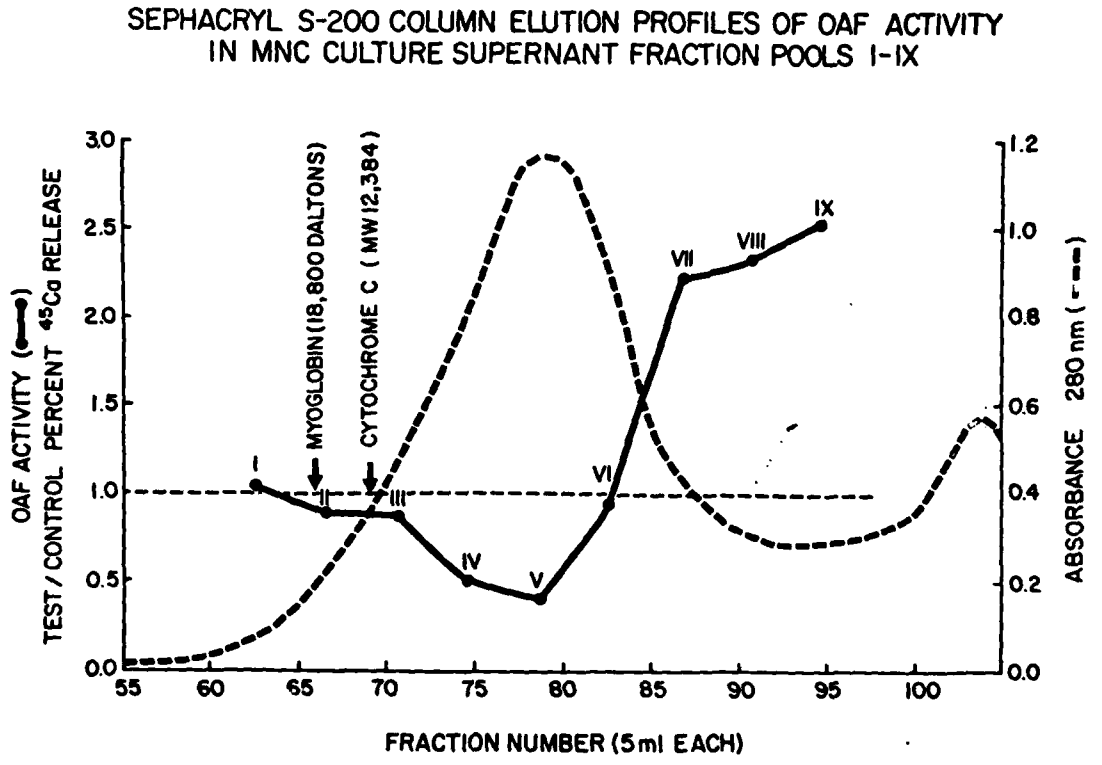

SEPHACRYL S-200 COLUMN ELUTION PROFILES OF OAF ACTIVITY
IN MNC CULTURE SUPERNANT FRACTION POOLS I-IX
OAF ACTIVITY (●—●)
TEST / CONTROL PERCENT ^{45}Ca RELEASE
ABSORBANCE 280 nm (▪--▪)
MYOGLOBIN (18,800 DALTONS)
CYTOCHROME C (MW 12,384)
I
II
III
IV
V
VI
VII
VIII
IX
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
55
60
65
70
75
80
85
90
95
100
FRACTION NUMBER (5ml EACH)

Figure 7.

REFERENCES

Boyum, A.: Isolation of Mononuclear Cells and Granulocytes from Human Cells. Scand J Clin Lab Invest (suppl) 97:77-89, 1968.

Bradford, M.M.: A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binging. Anal Biochem 72:248-254, 1976.

Dixon, M.: A Nomogram for Ammonium Sulfate Solutions. Biochem J 54:457-458, 1953.

Farrar, J.J.: In Vitro Reconstitution of T Cell-Deficient Mouse Spleen Cells by Cocultivation with Human Lymphocytes. J Immunol 112:1613-1616, 1974.

Farrar, J.J.: The Xenogeneic Effect. I. Antigen and Mitogen Stimulated Human Lymphocytes Produce a Nonantigen Specific Factor Which Reconstitutes the Antibody Response of T Cell-Deficient Mouse Spleen Cells. J Immunol 115:1295-1300, 1975.

Holtrop, M.E., Raisz, L.G., and King, G.J.: The Response of Osteoclasts to Prostaglandin and Osteoclast Activating Factor as Measured by Ultra-Structural Morphometry. IN: Proceedings, Mechanisms of Localized Bone Loss. Eds: Horton, Tarpley, and Davis. Special Suppl to Calcif Tissue Abstr pp 13-20, 1978.

Horton, J.E., Koopman, W.J., Farrar, J.J., Fuller-Bonar, J., and Mergenhagen, S.E.: Partial Purification of a Bone-Resorbing Factor Elaborated from Human Allogeneic Cultures. Cell Immunol 43:1-10, 1979.

Horton, J.E., Oppenheim, J.J., and Mergenhagen, S.E.: A Role for Cell Mediated Immunity in the Pathogenesis of Periodontal Disease. J Periodontol 45 (Part II): 351-360, 1974.

Horton, J.E., Oppenheim, J.J., Mergenhagen, S.E., and Raisz, L.G.: Macrophage Lymphocyte Synergy in the Production of OAF. J Immunol 113:1278-1287, 1974.

Horton, J.E., Raisz, L.G., Simmons, H.A., Oppenheim, J.J., and Mergenhagen, S.E.: Bone Resorbing Activity in Supernatant Fluid from Cultured Human Peripheral Blood Leukocytes. Science 177:793-795, 1 Sep 1972.

Ivanyi, L., Wilton, J., and Lehner, T.: Cell Mediated Immunity in Periodontal Disease, Cytotoxicity, Migration Inhibition, and Lymphocyte Transformation Studies. Immunology 22:141-145, 1972.

Klein, D.C. and Raisz, L.G.: Prostaglandins: Stimulation of Bone Resorption in Tissue Culture. Endocrinology 97:995-999, 1970.

Lamb, L., Hawley, C.E., and Kakari, S.: Effect of Fetal Calf Serum on the Production of Osteoclast Activating Factor in Mixed Leukocyte Cultures. J Dent Res 59A:333 (#264), 1980.

Lang, N. and Smith, F.: Lymphocyte Blastogenesis to Plaque Antigens in Human Periodontal Disease. I. Populations of Varying Severity of Disease. J Periodont Res 12:298-309, 1977.

Luben, R.A.: Purification of a Lymphokine: Osteoclast Activating Factor from Human Tonsil Lymphocytes. Biochem Biophys Res Commun 84:15-22, 1978.

Luben, R.A.: An Assay for Osteoclast Activating Factor (OAF) in Biological Fluids: Detection of OAF in the Serum of Myeloma Patients. Cell Immunol: 49:74-80, 1980.

Luben, R.A., Mohler, M.A., and Nedwin, G.E.: Production of Hybridomas Secreting Monoclonal Antibodies Against the Lymphokine Osteoclast Activating Factor. J Clin Invest 64:337-341, 1979.

Luben, R.A., Mundy, G.R., Trummel, C.L., and Raisz, L.G.: Partial Purification of OAF from PHA Stimulated Human Leukocytes. J Clin Invest 53:1473-1480, 1974.

Mackler, B., Altman, L., Wald, S., Rosenstreich, D., Oppenheim, J.J., and Mergenhagen, S.E.: Blastogenesis and Lymphokine Synthesis by T and B Lymphocytes from Patients with Periodontal Disease. Infect Immun 10: 10:844-850, 1974.

Mundy, G.R. and Raisz, L.G.: Big and Little Forms of Osteoclast Activating Factor. J Clin Invest 60:122-128, 1977.

Orczyk, G.P. and Behrman, H.R.: Ovulation Blockade by Aspirin and Indomethacin - In Vivo Evidence for a Role of Prostaglandin in Gonadotropin Secretion. Prostaglandins 1:3-20, 1972.

Page, R.C. and Schroeder, H.: Pathogenesis of Inflammatory Periodontal Disease. Lab Invest 33:235-241, 1976.

Patters, M., Genco, R., Reed, M., and Mashimo, P.: Blastogenic Response to Oral Bacteria Antigens: Comparison of Individuals with Periodontal Disease to Normal and Edentulous Subjects. Infect Immun 14:1213-1220, 1976.

Raisz, L.G., Luben, R.A., Mundy, G.R., Dietrich, J.W., Horton, J.E., and Trummel, C.L.: Effect of Osteoclast Activating Factor from Human Leukocytes on Bone Metabolism. J Clin Invest 56:408-413, 1975.

Raisz, L.G. and Niemann, I.: The Effects of Phosphate, Calcium, and Magnesium on Bone Resorption and Hormonal Responses in Tissue Culture. Endocrinology 85:446-452, 1969.

Seymour, G.J., Powell, R.N., and Davies, W.I.R.: The Immunopathogenesis of Progressive Chronic Inflammatory Periodontal Disease. J Oral Pathol 8: 249-265, 1979.

Trummel, C.L., Mundy, G.R., and Raisz, L.G.: Release of Osteoclast Activating Factor by Normal Peripheral Blood Leukocytes. J Lab Clin Med 85:1001-1007, 1975.

Wyan, V.J., Hawley, C.E., and Kakari, S.: An Evaluation of Concanavalin A (Con A) as a Stimulant for the Production of Osteoclast Activating Factor. J Dent Res 58A:320 (#912), 1979.

Yoneda, T. and Mundy, G.R.: Prostaglandins Are Necessary For Osteoclast Activating Factor Production by Activated Peripheral Blood Leukocytes. J Exp Med 149:279-283, 1979.

FORM A

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
and
AMERICAN ASSOCIATION FOR DENTAL RESEARCH

ABSTRACTS MUST BE RECEIVED BY OCTOBER 16, 1978

Complete: Items 1—5 below and type abstract within box, following instructions on reverse side.
Submit: Form A—Original and 4 Xerox copies; Form B—2 originals.

1. Full Name and Address of Author who will present paper:

V. J. Wyan
Division of Oral Biology
U. S. Army Institute of Dental Research
Walter Reed Army Medical Center
Washington, D. C. 20012

2. Mode of presentation:
- ☐ oral presentation only
- ☒ read by title acceptable
- ☐ poster presentation only
- ☒ oral or poster mode acceptable

3. *Do you wish to withdraw your paper if it*
- ☐ is placed in a mode not of your choosing?

GROUP CLASSIFICATION

4.

☐ Behavioral Sciences	☐ Neuroscience
☐ Cariology	☒ Periodontal Research
☐ Craniofacial Biology	☐ Pharmacology Therapeutics, Toxicology
☐ Dental Materials	
☒ Microbiology, Immunology	☐ Prosthodontics
☐ Mineralized Tissue	☐ Pulp Biology
	☐ Salivary Research

5. SUBJECT CLASSIFICATION

☐ Anatomy	☐ Nutrition
☐ Biochemistry	☐ Oral Medicine
☒ Cell Biology	☐ Oral Surgery
☐ Chemistry	☐ Orthodontics
☐ Education	☐ Pathology
☐ Embryology	☐ Physics
☐ Endocrinology	☐ Physiology
☐ Endodontics	☐ Preventive Dentistry
☐ Enzymology	☐ Radiology, Radiobiology
☐ Epidemiology-Biostatistics	☐ Restorative Dentistry
☐ Genetics	☐ Temporomandibular Joint
☐ Health Services	☒ Tissue Culture
☐ Histology	☐ Ultrastructure

☐ Other _____

TYPE PERFECT ORIGINAL OF ABSTRACT HERE
(Do not type beyond outline of box)

An Evaluation of Concanavalin A (Con A) As A Stimulant for the Production of Osteoclast Activating Factor. V. WYAN,* C. HAWLEY, and S. KAKARI. U.S. Army Institute of Dental Research, Washington, D. C.

Osteoclast Activating Factor (OAF) has been identified in mitogen stimulated leukocyte cultures. Con A was investigated here for its ability to stimulate OAF production in large quantities as required for OAF isolation. Human peripheral mononuclear cells (MNC) were cultured in RPMI 1640 containing 0.5% autologous plasma. Cultures were stimulated with Con A (10 µg/ml) after 24 hr, and the cells were labeled with tritiated thymidine (^{3}H TdR) 72 hr later. The ratio of stimulated to unstimulated ^{3}H TdR uptake ranged from 1.18 to 273. When the culture supernatants were evaluated in the bioassay for bone resorption, mean test over control percent ^{45}Ca release (T/C) ratios ranged from 0.88 to 1.85 in the small (1 ml) cultures and from 0.85 to 2.28 in large volume (>200 ml) cultures. The correlation coefficient between ^{3}H TdR uptake and T/C was $r = 0.31$ in small cultures ($p > 0.10$) and $r = 0.64$ ($p > 0.05$) in large cultures. There was a strong positive correlation, $r = 0.88$ ($p < 0.01$), between the T/C ratios produced by the small and large volume supernatants. These data show that OAF activity can be recovered from the large volume Con A stimulated MNC cultures. The OAF activity in these cultures is statistically similar to that shown in the 1 ml cultures ($p > 0.20$). However, the mitogenic response of MNC to Con A appears to be quantitatively independent of the OAF activity measured in the corresponding culture supernatants.

This investigation was supported by USPHS Inter Agency Agreement Y01-DE-60025 between U.S.A.I.D.R. and National Institute of Dental Research, Bethesda, Maryland.

31.

6. Reviewer's Rating:

☐	☐	☐	☐	☐
1	2	3	4	5

7. Disposition:

☐	☐	☐	☐	☐
O	P	T	R	W

FORM A
INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
and
AMERICAN ASSOCIATION FOR DENTAL RESEARCH

ABSTRACTS MUST BE RECEIVED BY OCTOBER 16, 1979

Complete: Items 1-7 below and type abstract within box, following instructions on reverse side.
Submit: Form A — Original and 4 Xerox copies; Form B card — 1 original (complete blank 3x5 card for each co-author, in same style as Form B).

1. Full name and address of author who will present paper.
LINDA LAMB
U. S. Army Institute of Dental Research
Walter Reed Army Medical Center
Washington, D. C. 20012

2. I wish my paper considered for (check one) IADR (Japan) ☐ AADR (Los Angeles) ☒

3. (For NIDR Grantees ONLY.)
I wish to apply for a Travel Assistance Grant for the IADR meeting in Japan. (See separate instructions.)
yes ☐ no ☐

If I do not receive a grant, I wish to submit my paper to AADR (Los Angeles). yes ☐ no ☐

4. Mode of presentation:
- ☐ oral presentation only
- ☐ read by title acceptable
- ☐ poster presentation only
- ☒ oral or poster mode acceptable

5. Do you wish to withdraw your paper if it is placed in a mode not of your choosing?
yes ☐ no ☒

6. **GROUP CLASSIFICATION**
- ☐ Behavioral Sciences
- ☐ Cariology
- ☐ Craniofacial Biology
- ☐ Dental Materials
- ☒ Microbiology, Immunology
- ☐ Mineralized Tissue
- ☐ Neuroscience
- ☐ Oral & Maxillofacial Surgery
- ☐ Periodontal Research
- ☐ Pharmacology, Therapeutics & Toxicology
- ☐ Prosthodontics
- ☐ Pulp Biology
- ☐ Salivary Research

7. **SUBJECT CLASSIFICATION**
- ☐ Anatomy
- ☐ Biochemistry
- ☐ Cell Biology
- ☐ Chemistry
- ☐ Education
- ☐ Embryology
- ☐ Endocrinology
- ☐ Endodontics
- ☐ Enzymology
- ☐ Epidemiology-Biostatistics
- ☐ Genetics
- ☐ Health Services
- ☐ Histology
- ☐ Nutrition
- ☐ Oral Medicine
- ☐ Orthodontics
- ☐ Pathology
- ☐ Physics
- ☐ Physiology
- ☐ Preventive Dentistry
- ☐ Radiology, Radiobiology
- ☐ Restorative Dentistry
- ☐ Temporomandibular Joint
- ☒ Tissue Culture
- ☐ Ultrastructure
- ☐ Other _____

TYPE PERFECT ORIGINAL OF ABSTRACT HERE
(Do not type beyond outline of box.)

Effect of Fetal Calf Serum on the Production of Osteoclast Activating Factor in Mixed Leukocyte Cultures. L. LAMB*, C. E. HAWLEY, & S. KAKARI. U. S. Army Institute of Dental Research, Walter Reed Army Medical Center, Washington, D. C.

Mixed leukocyte culture (MLC) supernatants free of extraneous proteins may facilitate the isolation and purification of osteoclast activating factor (OAF). Phytohemagglutinin stimulated mononuclear cell cultures (MNC) and mitogen-free MLC were incubated in media (RPMI-1640) alone or modified with 0.5%, 1.0%, or 2.0% fetal calf serum (FCS) or heat inactivated FCS (FCS*). Supernatants were tested for OAF activity by determining the ratio of test over control (T/C) percent ^{45}Ca release and blastogenesis by ^{3}H-thymidine ratio in stimulated and unstimulated cultures (MSI). The mean MSI and T/C ratios for MNC were 37.53±3.58 and 1.86±0.15, respectively. The mean MSI and T/C ratios for MLC without FCS were 2.84±0.96 and 1.10±0.05. With the addition of 0.5%, 1.0%, and 2.0% FCS, the mean MSI's were 3.30±0.80, 4.60±1.63, and 4.00±0.63. Corresponding mean T/C ratios were 1.08±0.06, 0.93±0.04, and 1.20±0.10. The mean MSI for MLC with 0.5%, 1.0% or 2.0% FCS* were 5.44±1.62, 4.20±1.02, and 8.25±2.53. Corresponding mean T/C ratios were 1.59±0.10, 1.72±0.12, and 1.75±0.12. The t-test showed no statistical difference ($p>0.10$) between the MSI of MLC using either FCS or FCS*. However, the OAF activity was higher ($p<.005$) when FCS* was used. These results show that OAF was produced only in MLC containing FCS* and these data indicate that OAF production was dependent upon FCS* in MLC and probably independent of blastogenesis.

This investigation was supported by USPHS Interagency Agreement YO1-DE-60025 between U. S. Army Institute of Dental Research and National Institute of Dental Research, Bethesda, MD.

8. Reviewer's Ratings:
32. ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5

9. Disposition:
☐ O ☐ P ☐ T ☐ R ☐ W

FORM A
INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
and
AMERICAN ASSOCIATION FOR DENTAL RESEARCH

ABSTRACTS MUST BE RECEIVED BY OCTOBER 16, 1979

Complete: Items 1-7 below and type abstract within box, following instructions on reverse side.
Submit: Form A — Original and 4 Xerox copies; Form B card — 1 original (complete blank 3x5 card for each co-author, in same style as Form B).

1. Full name and address of author who will present paper. Dr. Sophia Kakari
U. S. Army Institute of Dental Research
Walter Reed Army Medical Center
Washington, D. C. 20012

2. I wish my paper considered for (check one) IADR (Japan) ☐ AADR (Los Angeles) ☒

3. (For NIDR Grantees ONLY.)
I wish to apply for a Travel Assistance Grant for the IADR meeting in Japan. (See separate instructions.)
yes ☐ no ☐

If I do not receive a grant, I wish to submit my paper to AADR (Los Angeles). yes ☐ no ☐

4. Mode of presentation:
- ☐ oral presentation only
- ☐ read by title acceptable
- ☐ poster presentation only
- ☒ oral or poster mode acceptable

5. Do you wish to withdraw your paper if it is placed in a mode not of your choosing?
yes ☐ no ☒

6. **GROUP CLASSIFICATION**

☐ Behavioral Sciences	☐ Oral & Maxillofacial Surgery
☐ Cariology	☒ Periodontal Research
☐ Craniofacial Biology	☐ Pharmacology, Therapeutics & Toxicology
☐ Dental Materials	
☒ Microbiology, Immunology	☐ Prosthodontics
☒ Mineralized Tissue	☐ Pulp Biology
☐ Neuroscience	☐ Salivary Research

7. **SUBJECT CLASSIFICATION**

☐ Anatomy	☐ Nutrition
☐ Biochemistry	☐ Oral Medicine
☐ Cell Biology	☐ Orthodontics
☐ Chemistry	☐ Pathology
☐ Education	☐ Physics
☐ Embryology	☒ Physiology
☐ Endocrinology	☐ Preventive Dentistry
☐ Endodontics	☐ Radiology, Radiobiology
☐ Enzymology	☐ Restorative Dentistry
☐ Epidemiology-Biostatistics	☐ Temporomandibular Joint
☐ Genetics	☒ Tissue Culture
☐ Health Services	☐ Ultrastructure
☐ Histology	
☐ Other _____	

TYPE PERFECT ORIGINAL OF ABSTRACT HERE
(Do not type beyond outline of box.)

Suppression of Lymphokine Production by Aprotinin. KAKARI, S.*, LAMB, L. and HAWLEY, C.E. U. S. Army Institute of Dental Research, Walter Reed Army Medical Center, Washington, DC

Osteoclast activating factor (OAF), the lymphokine implicated in bone resorption of osteolytic disease processes, can also be generated *in vitro* by mitogen (i.e. phytohemagglutinin, PHA) stimulated normal human mononuclear cell (MNC) cultures. MNC mitogenic response was assessed by DNA synthesis using the ^{3}H-thymidine (^{3}H-TdR) uptake after 72 hours culture in RPMI-1640 with or without the protease-inhibitor Aprotinin (Trasylol) (20-1000 units/10^{6} cells) added, to possibly protect the protease-sensitive OAF molecule. OAF activity, estimated as the ratio of test/control percent ^{45}Ca released from prelabeled long fetal rat bones in organ cultures, was depressed in the MNC culture supernatants containing Aprotinin. This diminution in OAF yield was accompanied by a severely suppressed ^{3}H-TdR uptake. The observed decrease in OAF production may be related to the suppressed DNA synthesis of the MNC cultures. Whatever the mechanism of these effects, Aprotinin may offer a potential agent for the prevention of osteolytic processes, as in other tissue injuries.

This investigation was supported by USPHS Inter-Agency Agreement YO1-DE-60025 between U. S. Army Institute of Dental Research and National Institute of Dental Research, Bethesda, MD.

8. Reviewer's Ratings: ☐ 1. ☐ 2 ☐ 3 ☐ 4 ☐ 5
33.

9. Disposition: ☐ O ☐ P ☐ T ☐ R ☐ W

FORM A
INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
and
AMERICAN ASSOCIATION FOR DENTAL RESEARCH
ABSTRACTS MUST BE RECEIVED BY OCTOBER 16, 1979

Complete: Items 1-7 below and type abstract within box, following instructions on reverse side.
Submit: Form A — Original and 4 Xerox copies; Form B card — 1 original (complete blank 3x5 card for each co-author, in same style as Form B).

1. Full name and address of author who will present paper.
Dr. Sophia Kakari
U. S. Army Institute of Dental Research
Walter Reed Army Medical Center
Washington, D. C. 20012, U.S.A.

2. I wish my paper considered for (check one) IADR (Japan) ☒ AADR (Los Angeles) ☐

3. (For NIDR Grantees ONLY.)
I wish to apply for a Travel Assistance Grant for the IADR meeting in Japan. (See separate instructions.)
yes ☒ no ☐

If I do not receive a grant, I wish to submit my paper to AADR (Los Angeles). yes ☐ no ☒

4. Mode of presentation:
- ☐ oral presentation only
- ☐ read by title acceptable
- ☐ poster presentation only
- ☒ oral or poster mode acceptable

5. Do you wish to withdraw your paper if it is placed in a mode not of your choosing?
yes ☐ no ☒

6. **GROUP CLASSIFICATION**

☐ Behavioral Sciences	☐ Oral & Maxillofacial Surgery
☐ Cariology	☒ Periodontal Research
☐ Craniofacial Biology	☐ Pharmacology, Therapeutics & Toxicology
☐ Dental Materials	
☒ Microbiology, Immunology	☐ Prosthodontics
	☐ Pulp Biology
☒ Mineralized Tissue	☐ Salivary Research
☐ Neuroscience	

7. **SUBJECT CLASSIFICATION**

☐ Anatomy	☐ Nutrition
☒ Biochemistry	☐ Oral Medicine
☐ Cell Biology	☐ Orthodontics
☐ Chemistry	☐ Pathology
☐ Education	☐ Physics
☐ Embryology	☐ Physiology
☐ Endocrinology	☐ Preventive Dentistry
☐ Endodontics	☐ Radiology, Radiobiology
☐ Enzymology	☐ Restorative Dentistry
☐ Epidemiology-Biostatistics	☐ Temporomandibular Joint
☐ Genetics	☐ Tissue Culture
☐ Health Services	☐ Ultrastructure
☐ Histology	
☒ Other Immunology	

TYPE PERFECT ORIGINAL OF ABSTRACT HERE
(Do not type beyond outline of box.)

Generation and Physicochemical Characterization of Human Osteoclast Activating Factor. KAKARI, S.* and HAWLEY, C. E. U. S. Army Institute of Dental Research, Walter Reed Army Medical Center, Washington, D. C. 20012

The Lymphokine Osteoclast Activating Factor (OAF) has been implicated in the irreversible loss of alveolar bone in osteolytic disease. Human OAF is elaborated *in vitro* by dental plaque antigen - or mitogen-stimulated lymphocyte cultures. For OAF isolation and purification, generation of mg quantities of this protein are required. High OAF yields were obtained by phytohemagglutinin stimulated normal human peripheral mononuclear cell (MNC) cultures. MNC blastogenesis was ascertained by ^{3}H-thymidine (^{3}H TdR) uptake after incubation for 72hrs/37C in RPMI-1640. OAF activity in the cell-free culture supernatants and their purification fractions were monitored in the bioassay for bone resorption by the test/control ratio of percent ^{45}Ca release from fetal rat long bones. The Um-2 membrane - concentrated and partially purified MNC supernatants were chromatographed through Sephacryl S-200 (Pharmacia). Concentrated pooled active column fractions (MW 12,000 - 25,000 Daltons) were further analyzed by High Performance Liquid Chromatography (HPLC) using protein analysis columns for size exclusion (Waters Assoc. Inc.). OAF activity was recovered in the MW range of 12,000 - 21,000 Daltons. The chromatographic fractions were further analyzed by polyacrylamide gel electropheresis and isoelectric focusing. This is the first report on the potential use of HPLC in the purification and physicochemical characterization of OAF.

This study was supported by USPHS Interagency Agreement YO1-DE-60025 between U.S.A.I.D.R. and National Institute of Dental Research, Bethesda, MD

8. Reviewer's Ratings:
34. ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5

9. Disposition:
☐ O ☐ P ☐ T ☐ R ☐ W

Official Abstract Form

(Read all instructions before typing)

Start→ Partial Purification and Characterization of Osteoclast Activating Factor by High Performance Liquid Chromatography. J. R. Heath* and C. E. Hawley. U. S. Army Institute of Dental Research, Walter Reed Army Medical Center, Washington, D. C.

The lymphokine osteoclast activating factor (OAF) was produced in phytohemagglutinin stimulated large volume cultures of human mononuclear leukocytes (MNL). OAF activity was measured in a bioassay for bone resorption by the test/control ratios of per cent ^{45}Ca release from paired fetal rat bones. OAF in MNL culture supernatants was concentrated and partially purified by ultra filtration. The concentrated supernatants were fractionated by gel filtration on Sephacryl S-200 columns or by precipitation with increasing concentrations of $(NH_4)_2SO_4$. OAF active fractions were pooled, concentrated, and analyzed by high performance liquid chromatography (HPLC) using protein analysis columns. Bioassays of HPLC fractions of OAF active S-200 pools showed isolated OAF activity at 18,000 daltons while bioassays of chromatographed OAF active $(NH_4)_2SO_4$ fractions (80-100% saturation) showed OAF activity at 18,000 and 9,000 daltons. Data indicates that there was a partial dissociation of the 18,000 dalton OAF during salt fractionation and verifies the 9,000 dalton species as the basic subunit of OAF. HPLC has been shown to be a reliable tool in lymphokine purification.

☐ A
☐ R
☐ PS

INSTRUCTIONS

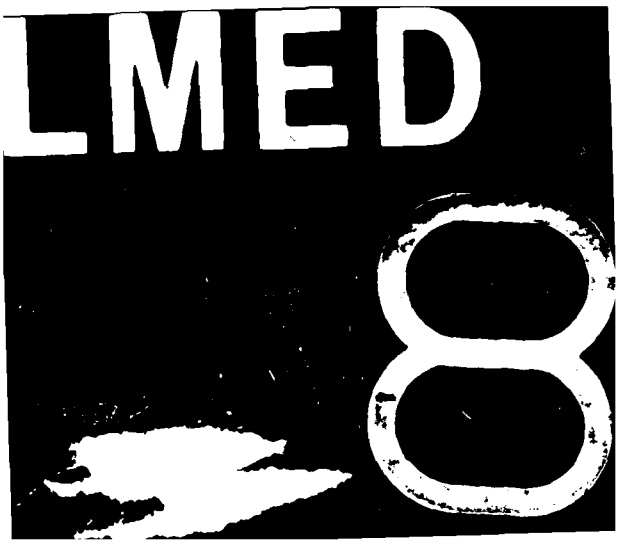
Indicate below the subject category number from the list on p. vi; check your Poster/Slide Session preference; complete the check list on the reverse side of this sheet; and sign your name in the space provided.

Indicate category number from page vi. 31
Category number

POSTER/SLIDE SESSION PREFERENCE

Because of the flexibility in programming afforded by Poster Sessions, the Program Committee will attempt to schedule *all* abstracts which (a) are considered by elected divisional officers to be of acceptable quality and (b) which conform to rules established by the Program Committee. This will result in the elimination of "Read-by-Title" papers. The decision as to whether an abstract is scheduled in a "Slide" or a Poster Session will be made by the elected Program Committee which will be guided (but not bound) by the preference of the authors. The decision will depend on the nature of the subject matter, the numbers of related abstracts, the availability of session rooms of appropriate size, avoidance of conflicting sessions and events, and other such considerations. Approximately 60 per cent of the abstracts will be scheduled in poster sessions. By submitting an abstract, the author agrees that the paper will be presented as scheduled.

I prefer presentation in a Poster Session. ☐
I prefer presentation in a Slide Session. ☐
I have no preference. ☒


LMED
8